[CANCER RESEARCH 40, 4694–4703, December 1980]
0008-5472/80/0040-0000$02.00

A Survey of Transformation Markers in Differentiating Epidermal Cell Lines in Culture[1]

Stuart H. Yuspa,[2] Pamela Hawley-Nelson, Barbara Koehler, and John R. Stanley[3]

In Vitro Pathogenesis Section, Laboratory of Experimental Pathology [S. H. Y., B. K.] and Dermatology Branch [J. R. S.], National Cancer Institute; Laboratory of Developmental Biology and Anomalies, National Institute of Dental Research [J. R. S.]; and Microbiological Associates [P. H. N.], Bethesda, Maryland, 20205

ABSTRACT

Primary mouse epidermal cells underwent spontaneous malignant transformation in culture. Twelve malignant epidermal cell lines were established which produced squamous cell carcinomas in syngeneic hosts. These lines were used to define criteria for recognizing transformed epidermal cells *in vitro*. Growth in suspension in agar, agarose, or Methocel was minimal for 11 of the lines. All lines tested retained specific epidermal antigens (pemphigus, pemphigoid, keratin) by indirect immunofluorescence, but keratin content was reduced when quantified by radioimmunoassay. Basal activity of ornithine decarboxylase and activity induced by the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate were variable among lines. All malignant lines as well as normal epidermal cells grew well at reduced extracellular calcium concentrations. When the extracellular calcium was elevated, normal cells ceased proliferation, terminally differentiated, and sloughed from the culture dish, while malignant cells continued to proliferate although they expressed differentiative functions. These results indicate that malignant transformation in epidermis is associated with a fundamental alteration in the program of terminal differentiation which allows some cells to escape the proliferative block and cell death which accompanies differentiation in normal keratinocytes. This alteration should be useful to select for transformants during the process of carcinogenesis *in vitro*.

INTRODUCTION

The establishment of criteria for the recognition of malignant transformation is essential for complete development of *in vitro* models to study carcinogenesis. This laboratory utilizes mouse epidermal cells in culture for a variety of studies related to differentiation and carcinogenesis (43, 46). Early in the development of this culture system, we reported that morphological criteria would not suffice as a reliable distinguishing characteristic of malignant epidermal cells in culture (12, 47). These findings have been confirmed by other laboratories which utilize epidermal cells (6, 15, 35). The lack of characteristic morphological changes *in vitro* associated with malignant transformation appears to be common to a variety of other epithelial cell types (25, 27, 32, 40). This is in contrast to mesenchymal cell transformation models where morphological alterations are strongly associated with malignant potential (10, 19).

Recent advances in culture techniques for mouse epidermal cells have provided a new approach to the establishment of a quantitative transformation assay *in vitro* (21, 24, 46). Modifications of standard culture procedures have also resulted in the evolution of continuously growing epidermal cell lines with morphological and biochemical characteristics of normal keratinocytes. These lines are tumorigenic when transplanted to syngeneic hosts. The availability of many such cell lines has provided an opportunity to reexamine the phenotype of differentiating malignant keratinocytes. This report presents an analysis of biological, biochemical, and pharmacological markers studied in order to establish an association with epidermal transformation.

MATERIALS AND METHODS

Development of Cell Lines. The isolation and cultivation of keratinocytes from newborn BALB/c mice have been described previously (44). Cells were plated at 10^5/sq cm onto 60-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and grown at 37° with twice-weekly medium changes. The method was altered only in that cells were plated in Eagle's minimal essential medium (Grand Island Biological Company, Grand Island, N. Y.) enriched 5-fold with essential and nonessential amino acids and vitamins similar to the medium described by Fusenig and Worst (17). Fetal calf serum (Reheis Chemical Company, Kankakee, Ill.) and antibiotics (Antibiotic-Antimycotic Solution; Grand Island Biological Company) were added at 14% and 1%, respectively. In this medium, mouse epidermal cells proliferated, stratified, and terminally differentiated as described previously (11, 43, 44). Following a wave of differentiation, large cells with poorly distinguished borders were the predominant population as reported for Medium 199 (44). However, the retained cell density was considerably higher in the enriched medium following the initial sloughing of differentiated cells. By the fourth week in culture, small dense cellular foci were observed (usually 1 to 2 per 60-mm dish) which were easily identified because they continued to stratify and were covered with a keratin sheet.

These epithelial foci were found to resist mild trypsin treatment while the nonstratifying cell population could be removed by such treatment. Thus, when cultures were exposed to a film of 0.1% trypsin in Hanks' balanced salt solution for 10 min at 37°, nonstratifying cells became rounded and were washed off with phosphate-buffered saline (NIH Media Unit; 0.14 M NaCl: 2.0 mM KCl:8.3 mM Na_2HPO_4:1.4 mM KH_2PO_4). This procedure was repeated at intervals over many months until the stratifying colonies became dominant and finally the only cell type in each dish. Once established (a period of 6 to 8 months), these differentiating epithelial cell lines were switched to standard Eagle's minimal essential medium plus 10% fetal calf serum and could be subcultured by the use of 0.05% trypsin:0.1% EDTA. Using these procedures, a total of 13 cell lines from 2 separate primary epidermal isolations were developed. These

[1] Portions of this work were performed under NCI Contract NO1-CP-02199.
[2] To whom requests for reprints should be addressed.
[3] Intergovernmental Personnel Act (IPA) Visiting Scientist from the Department of Dermatology, New York University Medical Center, New York, N. Y.
Received June 24, 1980; accepted September 16, 1980.

were designated Pam 1 and Pam 21 to 29, 210 to 212. This paper will discuss results obtained with 12 of these lines developed from a single isolate (Pam 21 to 29, 210 to 212) of keratinocytes from newborn BALB/c mice. The 12 lines were derived from foci in 4 different primary plates which were subcultured into 24 wells of a multiwell culture dish. The lines have not been cloned but have been maintained as independent lines developing in a particular well from the time of the first subculture.

Biological Assays. Tumorigenicity was determined by injecting 2×10^6 cells in 0.1 ml of phosphate-buffered saline in the interscapular region of newborn syngeneic mice. After weaning (21 days), animals were examined every week or biweekly, and tumors were excised when they were 1 cm or more in diameter. All tumors were fixed in formalin and examined histologically. Anchorage-independent growth was determined in 0.33% agar (Bacto-Agar; Difco Laboratories, Detroit, Mich.) (5), 0.33% agarose (Seakem Agarose; Microbiological Associates, Bethesda, Md.) (30), or 1.5% methylcellulose (Methocel, 4000 centipoise, Fisher Scientific Company, Fairlawn, N. J.) (34) by plating 10^4 cells in each suspending medium over a 1% agar base as described previously (5). Preparation of media with a lowered calcium content has been reported (21). Cells used as positive controls for anchorage-independent growth assays were the malignant lines EC-1 described by this laboratory (47) and T^36272, a derivative of line T62 which has been described previously (5), kindly supplied by Dr. Nancy Colburn of the National Cancer Institute.

Assays for Specific Chemical Markers. Cell extracts were prepared and analyzed on 9% polyacrylamide gels (containing 0.1% sodium dodecyl sulfate) as described in a previous report for primary cultures (38). Extracts prepared in a similar way were analyzed for mouse keratin content by a radioimmunoassay developed in this laboratory (48).

Assays for Specific Immunological Markers. Sera from patients with bullous pemphigoid and pemphigus vulgaris contain antibodies against an epidermal basement membrane antigen and a keratinocyte cell surface antigen, respectively (36). These antibodies are known to react with their respective epidermal antigens across species lines (9). Four bullous pemphigoid sera, 3 pemphigus sera, and 5 normal human sera were used for IIF[4] studies. Pemphigoid and pemphigus sera had antibody titers of greater than 1:40 on cryostat sections of normal human skin. Antisera from rabbits against the mouse keratin proteins K_1 (M.W. 68,000) and K_2 (M.W. 60,000) were prepared in this laboratory as reported previously (39). Antiserum from rabbits directed against the M.W. 58,000 intermediate filament protein of CHO cells (2) was kindly supplied by Drs. Michael Gottesman and Fernando Cabral of the National Cancer Institute, Bethesda, Md. Three normal rabbit sera, including a preimmune serum from a rabbit subsequently immunized to keratin, served as negative controls.

IIF was performed on cell cultures by a previously described procedure (36) with minor modifications. In brief, cells grown on cover slips were fixed in −20° methanol for 10 min and then −20° acetone for 10 sec. In studies designed to visualize keratin fibers, fixed coverslips were treated with 0.5% Triton X-100 in 0.01 M Tris-buffered 0.85% NaCl solution (pH 7.4) for 30 min at room temperature. In order to reduce nonspecific fluorescence, 4% bovine serum albumin in 0.02 M phosphate-buffered saline was used to dilute all sera and to wash the cells after the sera were applied, and the fixed cells were incubated initially for 30 min with a normal goat serum at a dilution of 1:10. The second serum applied was bullous pemphigoid, pemphigus, normal human serum, rabbit antiserum to keratin (anti-K_1 or anti-K_2), antiserum to the CHO intermediate filament, or normal rabbit serum. The human sera were diluted 1:5 or 1:10; the rabbit sera were diluted 1:20 or 1:80. A fluorescein isothiocyanate-conjugated goat anti-human IgG or goat anti-rabbit IgG antibody (Cappel Laboratories, Downington, Pa.) diluted 1:40 was then applied. The same IIF procedure was used on 4- to 6-μm cryostat sections of tumor tissue embedded and frozen in Tissue-Tek II O.C.T. compound (Lab-Tek Products, Naperville, Ill.), except that tissue sections were air dried and not fixed in methanol or acetone. The cells or tissue sections were viewed with a Leitz Ortho II epiilluminated fluorescent microscope.

Assays for Pharmacological Response. Cell lines were exposed to medium containing TPA (0.1 μg/ml); (Chemical Carcinogenesis, Eden Prairie, Minn.) or solvent alone (0.1% dimethyl sulfoxide). The activity of the enzyme ODC was measured at 4 and 8 hr after exposure as described previously (26).

RESULTS

Characteristics of Cells and Tumors. All cell lines retained many morphological characteristics of primary epidermal cells in culture. Cells were round to polygonal and tended to grow in colonies as a monolayer when plated sparsely and to stratify in a focal or diffuse fashion when grown to confluence (Fig. 1). Differentiating cells often contained granules. Patterns of differentiation varied from line to line but often more than one pattern was seen in different regions of the same line. In general, the keratinization patterns reported by Indo and Miyaji (22) for transformed rat epidermal cells were also observed for these mouse cell lines. Examples of these patterns are seen in Fig. 1. Electron micrographs of several lines revealed typical epidermal markers such as desmosomes, tonofilaments, keratohyalin granules, and in cross-sections a vertical maturation pattern.

All lines tested for their ability to produce tumors in newborn syngeneic hosts proved positive (Table 1). Injections were first performed at passages 5 to 7, at which time the cells had been in culture for over 1 year. All tumors were squamous cell carcinomas. Tumors from most individual lines were either well differentiated or poorly differentiated, but some were mixed. Most tumors from a particular line were morphologically similar, but exceptions were observed. No strict correlation between *in vitro* morphology of cells and the histological tumor type could be made. That is, cell lines which exhibited considerable differentiative function *in vitro* (such as line 26) gave rise to poorly differentiated tumors. There also was no correlation between histological tumor type and the time of first appearance of a tumor or how rapidly it grew. For example, tumors derived from Pam 212 were very well-differentiated but had the shortest latency period, a 100% incidence, and grew rapidly.

Anchorage-independent Growth. Previous studies with transformed mouse epidermal cells in which differentiative function was not strongly expressed had indicated that the

[4] The abbreviations used are: IIF, indirect immunofluorescence; CHO, chinese hamster ovary; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase.

Table 1
Tumorigenicity of Pam lines in syngeneic hosts

Cell line[a]	No. of tumors/ no. of mice injected	Time to first tumor/time to last tumor (days)	Predominant histological tumor type
21	13/13	48/105	Keratinizing squamous cell carcinoma
22	10/17	105/351	Poorly differentiated squamous cell carcinoma
23	9/12	70/351	Poorly differentiated squamous cell carcinoma
24	ND[b]	60/88	
25	9/9		Poorly differentiated squamous cell carcinoma
26	11/11	48/53	Poorly differentiated squamous cell carcinoma
27	7/7	39/39	Areas of well- and poorly differentiated squamous cell carcinoma
28	10/11	77/351	Poorly differentiated squamous cell carcinoma
29	8/12	77/105	Keratinizing squamous cell carcinoma
210	5/6	105/351	Keratinizing squamous cell carcinoma
211	15/15	48/60	Keratinizing squamous cell carcinoma
212	11/11	39/39	Keratinizing squamous cell carcinoma

[a] All lines were tested in passages 5 to 7 (more than 1 year in culture).
[b] ND, not determined.

Table 2
Anchorage-independent growth of transformed epidermal cells

			Colony-forming efficiency in the following media[a] (no. of colonies/10,000 cells plated) × 100		
Cell line	Passage	Tumorigenic	0.33% agar	0.33% agarose	1.5% Methocel
Pam 21	5	+	<1	ND	ND
	7-9		<1	2	1.6
Pam 22	5	+	<1	ND	ND
	11-13		1.4	1.7	<1
Pam 23	5	+	1.2	ND	ND
Pam 24	5	ND[b]	<1	ND	ND
Pam 25	6	+	<1	ND	ND
Pam 26	6	+	<1	ND	ND
Pam 27	5-7	+	3.4[c]	ND	ND
Pam 28	5-7	+	<1	ND	ND
Pam 29	5	+	2.0	ND	ND
Pam 210	5	+	ND	ND	ND
Pam 211	5	+	<1	ND	ND
	10-12		<1	<1	<1
Pam 212	6-8	+	<1	<1	<1
	6-8	+	<1		
	16-18	+	<1		
Pam 212T[d]			16.5[c]		
Pam 27T[d]			3.3[c]		
EC-1		+	4[c]		
$T^3$6272		+	29[c]	31[c]	7.3[c]
Keratinocytes	Primary	−	0	ND	ND

[a] All assays were performed by plating 10^4 cells in the appropriate suspending medium prepared in Eagle's minimal essential medium containing 20% fetal calf serum. Parallel assays in agar utilizing 10% serum failed to produce any colonies from Pam lines. All colonies were smaller than 10 cells and in most cases contained 4 to 6 cells. A value of <1 indicates a range of 0 to 99 small colonies per plate. Most lines yielded 15 to 50 colonies in repeated tests.
[b] ND, not determined.
[c] Colonies considerably larger (100 cells).
[d] Cell lines established from tumors excised from animals receiving Pam 212 or 27 cells.

ability to grow in suspension in agar was a reliable criterion for tumorigenicity (6, 35). Fusenig *et al.* (15) reported a similar finding for more-differentiated malignant epidermal cell lines. However, differentiating malignant rat epidermal cells failed to form colonies in agar (22). Studies with Pam lines indicate that plating in 0.33% agar produces only a small number of colonies, and colonies were generally less than 10 cells (Table 2). It cannot be said with certainty that such colonies were viable and expanding. Only line 27 produced a significant number of larger colonies in agar. In contrast, 2 tumorigenic lines which are derived from epidermal cells but do not differentiate in culture to the same extent as Pam cells were used as positive controls and produced many large colonies in agar. Additional studies with lines 21, 22, 27, 211, and 212 at passages 7 or 11 indicated that the incorporation of the mitogens epidermal growth factor or TPA into the agar (5) failed to enhance colony formation. Likewise, reduction of calcium concentration in the agar failed to enhance growth of these lines (13). Of interest is the finding that colony-forming efficiency of Pam 212 was significantly increased when tumors derived from this line were regrown *in vitro* and tested in agar (Table 2). Whether this treatment selected cells with this potential or induced a change is not known. Cells derived in the same way from Pam 27 tumors did not demonstrate enhanced agar growth, but such cells were positive prior to tumor formation. Both tumor derived lines retained their differentiative characteristics. It has been reported that agarose (30) or Methocel (34) may be better media for testing growth in suspension for cells which have stringent growth characteristics. Table 2 reveals that these conditions failed to enhance colony-forming efficiency in any of the lines tested and that Methocel may have decreased the efficiency of $T^3$6272.

Induction of Terminal Differentiation. The discovery that ionic calcium is a critical regulator of growth and differentiation of epidermis *in vitro* has provided a means to select for cells with an altered capacity for terminal differentiation (20, 21, 24, 46). Normal epidermal cells proliferate rapidly in medium with a low ionic calcium concentration (0.2 to 0.09 mM) and terminally differentiate when calcium is elevated above 0.1 mM (20, 21). Since the transformed phenotype is likely to be associated with an alteration in differentiative function, the effect of calcium concentration was studied in Pam lines. When Pam 211 was grown in medium containing 0.07 mM Ca^{2+}, the cells grew as a monolayer without stratification (Fig. 2*a*). Individual cells were polygonal with distinct intercellular spaces, giving the cell sheet a paving stone appearance when confluent. Many round cells floated into the medium under these conditions. These results are identical to the growth pattern observed for normal epidermal cells grown in low-calcium medium (20, 21). When Pam 211 cells grown for 1 week at 0.07 mM Ca^{2+} were changed to medium with 1.2 mM Ca^{2+}, the cells became less refractile, and the size of intercellular spaces decreased after only a few hr (Fig. 2*b*). Individual cells became smaller, and the culture became more compact. Focal stratification was observed by 48 hr, but the cultures maintained a balance between proliferation and differentiation (Fig. 2*c*) which resulted in continuous growth as in Fig. 1. All other Pam lines responded identically to these culture conditions. This is in contrast to normal epidermal cells which stop proliferating and terminally differentiate *en mass* after a switch from low-calcium medium to medium with a higher calcium concentration. In cultures of normal cells, switching from 0.07 to 1.2 mM Ca^{2+} leads to death and sloughing of most of the cells (20, 21). Thus, malignant cells appear

to have an alteration in the normal controls which result in a calcium-induced proliferative block and cell death by terminal differentiation.

Markers of Epidermal Differentiation. Cultured mouse keratinocytes display many of the specific differentiation markers identified for epidermis *in vivo* (38, 39, 45). Pam lines were studied to determine if the alteration observed in the regulation of differentiation was accompanied by specific biochemical or immunological differences in differentiation markers. The study presented in Fig. 3 compares the electrophoretic separation of proteins from normal and malignant cells.

Previously, we had shown that mouse basal cells contain a major keratin protein with a molecular weight of 60,000 (K_2) and a minor keratin protein with a molecular weight of 68,000 (K_1). During differentiation, the K_1:K_2 ratio becomes unity, and this is the pattern observed in the mouse stratum corneum (38) where K_1 and K_2 represent virtually all of the keratin molecules. Mouse keratinocytes also contain significant amounts of actin, particularly in the basal cell population (Fig. 3, *a* and *b*). The major bands in the electrophoretic pattern of 4 Pam lines were qualitatively similar to those of the basal cell population (Fig. 3, *c* to *f*). There was a broadening in the K_2 region which might indicate a poorly resolved second band in that area, but a number of gel runs failed to yield a distinct reproducible change in pattern in that region. The actin band also appeared enriched in Pam lines, and an unidentified band (M.W. 30,000 to 35,000) may be increased. There also was an apparent decrease in smaller-molecular-weight proteins toward the front of the gel which were prominent in basal cells. Nevertheless, these studies did not reveal either a loss or a gain of a specific marker protein which characterized the transformed state.

Epidermal specific markers and other cellular antigens were studied by IIF as well. Previous studies had suggested that mouse and human squamous cell carcinomas had decreased or absent pemphigus and pemphigoid antigens (7, 8, 29). In contrast, our studies show these antigens were strongly expressed in the tumors derived from Pam 212 (Fig. 4, *a* and *b*). Furthermore, in cultured Pam cells, both pemphigoid and pemphigus antigen were readily demonstrated by IIF techniques (Fig. 4, *c* and *d*; Table 3). Pemphigus antigen was detected mainly on the cell membranes of the larger flat cells located more superficially in the culture, while pemphigoid antigen showed a pattern of coarsely granular fluorescence within or under the cytoplasm of many of the cells attached to the substrate. These antigens were as prominent in the cultured transformed lines as in the cultured normal mouse keratinocytes and were present in the same pattern as we have reported for cultured human epidermal cells (36). Cultured human dermal fibroblasts did not express either antigen, and no immunofluorescence was seen with normal human sera in Pam cells. All cultured Pam lines studied also demonstrated a prominent filamentous cytoskeletal network of keratin proteins (Fig. 4*e*).

No qualitative differences in the keratin pattern were observed between Pam lines and normal keratinocytes. However, when extracts of Pam 21, 22, 27, and 212 were assayed for keratin protein content by a quantitative radioimmunoassay (48), the transformed lines contained less than 20% of total keratin protein (expressed as μg keratin per mg total protein) assayed in normal primary keratinocytes. The keratin content of the 4 lines studied was quite constant by radioimmunoassay. Mouse dermal fibroblasts were negative for keratin by IIF and had negligible (nonspecific background) levels by radioimmunoassay. Studies from another laboratory had indicated that a spontaneously transformed mouse epidermal cell line synthesized a M.W. 58,000 protein which was a characteristic component of the filamentous cytoskeleton proteins of mesenchymal cells (14). Keratin cytoskeletal proteins were also coexpressed in this cell line. Pam cells were examined by IIF with an antibody raised against the M.W. 58,000 mesenchymal cytoskeletal protein of CHO cells (2). This protein is believed to be both biochemically and immunologically similar to the mouse 3T3 protein (2). Four Pam lines were negative with this antiserum, while mouse dermal fibroblasts displayed a prominent filamentous fluorescent pattern. Primary keratinocytes were also negative in this study, although contaminating melanocytes were positive.

Pharmacological Responsiveness. Some malignant cells demonstrate enhanced responsiveness to the potent tumor promoter TPA (41). We have been studying the induction of ODC in normal keratinocytes exposed to TPA (26). This enzyme is reported to be elevated in mouse skin tumors (31). It was therefore possible that either enhanced induction, or high basal levels of ODC might characterize malignant keratinocytes *in vitro*. Table 4 indicates that neither of these anticipated characteristics was consistently found in the Pam cells. Certain lines (lines 29 and 212) had high basal activity, and others showed exceptional inducibility (lines 24, 211, and 212); however, most lines were not significantly different from primary keratinocytes, and several were poor responders with low basal activity. Furthermore, activity and responsiveness varied in some lines between experiments; this variability could be related to the degree of confluency at the time of treatment. It

Table 3

IIF assays of malignant epidermal cells for normal epidermal and mesenchymal antigens

Assays were performed on fixed and permeabilized cultured cells or on cryostat sections of tumors.

	Sera tested					
Cell line	Pemphigus	Pemphigoid	Keratin	CHO intermediate filament	Normal rabbit serum	Normal human serum
Pam 211	+	+	+	−	−	−
Pam 212	+	+	+	−	−	−
Pam 21	ND[a]	ND	+	−	−	ND
Pam 22	ND	ND	+	−	−	ND
Tumor 212 (*in vivo*)	+	+	+	ND	−	−
Primary mouse epidermal cells	+	+	+	−	−	−
Primary dermal fibroblast	−	−	−	+	−	−

[a] ND, not determined.

Table 4
Basal and induced ornithine decarboxylase activity in transformed epidermal cells

Cell line	8-hr basal activity		8-hr induced activity	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Pam 21	0.07	0.09	0.38 (5.4)[a]	0.21 (2.3)
Pam 22	0.59	0.20	3.28 (5.6)	0.91 (4.6)
Pam 23	0.09	0.19	0.20 (2.2)	0.24 (1.26)
Pam 24	0.10	0.16	8.17 (82)	2.8 (17.5)
Pam 25	0.40	0.15	0.82 (2.1)	1.0 (6.7)
Pam 26	0.86	0.27	1.20 (1.4)	0.36 (1.3)
Pam 27	0.18	0.10	0.22 (1.2)	0.48 (4.8)
Pam 28	0.15	0.14	0.49 (3.3)	1.0 (7.1)
Pam 29	3.68	1.16	7.36 (2.0)	7.20 (6.2)
Pam 210	ND[b]	0.03	ND	0.35 (11.7)
Pam 211	ND	0.26	ND	6.0 (23)
Pam 212	1.91	0.08	44.62 (23.4)	4.6 (57.5)
Primary epidermal cells	0.3	0.4	2.4 (8)	4.6 (11.5)

[a] Numbers in parentheses, degree of induction. Slight variability is expected depending on confluency at time of treatment and on serum batch.
[b] ND, not determined.

was also possible that the kinetics of ODC induction after TPA was different in malignant cells and normal cells. Previously, we had shown that peak activity in normal cells occurs at 6 to 9 hr after exposure. However, cells were also assayed at 4 hr after TPA in the experiments shown in Table 4, and the results were essentially the same.

DISCUSSION

The availability of a number of tumorigenic epidermal cell lines which yield squamous cell carcinoma upon injection into syngeneic hosts has provided the means to assess the reliability of individual markers of epidermal transformation. The development of these lines has been very reproducible, and, consequently, other lines are currently becoming available to extend these studies to test additional markers. Pam 1 cells, derived in an identical way from a different primary cell isolate, have not been studied in great detail but resemble Pam 2 cells in morphology and in the production of squamous cell carcinoma upon reinjection to syngeneic recipients. Spontaneous transformation in mouse epidermal cells cultured for prolonged periods has been reported in several laboratories (6, 12, 16), but the establishment of well-differentiated lines has been rare. The techniques described here provide a way to routinely obtain such lines in the absence of mesenchymal influences. It is anticipated that, with slight alteration in the procedures described in this report, nonmalignant cell lines could be developed as well. Other techniques for establishing well-differentiated, nonmalignant epidermal cell lines have also been the subject of a previous report from this laboratory (46). These reported techniques are being used to develop a quantitative transformation assay for chemical carcinogens in epidermal cell culture (21, 24). The tendency for epidermal cells to undergo spontaneous neoplastic transformation will undoubtedly complicate this system. Epithelial cell cultures derived from other rodent tissues also have a tendency to spontaneously develop infinite life spans *in vitro* or to spontaneously transform (23, 42). This appears to be a property shared by epithelial and mesenchymal cells *in vitro*.

Morphological criteria have been uniformly poor for predicting cancer in epithelial cells, with the possible exception of careful cytological analysis of transformed hepatocytes (28). However, a cytological analysis makes the selection and quantitation of transformed clones very difficult and thus is useful only for identifying the presence of malignant cells in a population. Our studies indicate that morphological assessment of the degree of differentiation in a cell line (as evidenced by the capacity to stratify and produce squames) does not necessarily correlate with the histological differentiation found in the resultant tumor nor with the rapidity of tumor development and growth. Studies with Pam 211 and 212 have also indicated that a repeated subculture (>16 passages) can lead to an altered morphology with less-differentiated characteristics. However, these latter subcultures have not been extensively studied with the markers reported here.

The ability to grow in agar has been reported to be a reliable criterion as a marker for transformed epithelial cells of both epidermal (6, 15, 35) and nonepidermal (23, 32, 40) origin. However, our well-differentiated lines formed only a few small colonies in suspension in agreement with the findings of Indo and Miyagi (22) for transformed rat epidermal cells. Growth in other suspending media, reported to enhance expression of anchorage independence (30, 34), did not alter these results, but a variety of other agar modifications reported to enhance anchorage independence have not been tried. Suspension culture has been used as a method to induce terminal differentiation in normal human epidermal cells and epidermal-like cells derived from a mouse teratocarcinoma (18). Induction of terminal differentiation in this way could explain the inability of the cell lines reported here to form colonies in suspension and would imply different mechanisms involved in the induction of differentiation by suspension and Ca^{2+}. It is possible that a repeated subculture could lead to the acquisition of anchorage independence associated with the loss of differentiative characteristics. However, our data at present indicate that anchorage independence need not precede the ability to form tumors. The formation of epidermal cell colonies over an agar base (22) and in rotation culture (16) was reported to be characteristic of transformed lines. Pam lines have not yet been tested by this criterion. While only 2 lines derived from tumors produced by Pam cells have been tested for agar growth, in the case of Pam 212 anchorage independence developed. This could imply a selection of cells *in vivo* and indicate that a minor population exists within Pam lines which is responsible both for the formation of tumors and growth in agar. Alternatively, passage *in vivo* could induce a change which allows anchorage-independent growth.

It was hoped that changes in differentiation markers would provide tissue-specific criteria for acquisition of the malignant phenotype. Previous studies by Carruthers (3) had indicated that antigenic changes occurred during the process of epidermal carcinogenesis, although the specific antigens were not identified. Fusenig *et al.* (15, 16) had reported that reactivity to an antiserum raised against normal epidermal cell surface antigens was diminished in malignant epidermal cell lines. Similar studies by Colburn (4) could not confirm this as a consistent finding for malignant cells, although cell lines in general had reduced reactivity. Our studies with specific antigenic markers failed to define a change which was characteristic of transformation. Of interest was the finding that in tumors, pemphigoid antigen, found associated with the basement membrane in normal epidermis, was localized to the perimeter of tumor nests where the expanding cells were invading the host

stromal tissues. Also, pemphigoid antigen was found *in vitro* in the cells attached to the plastic surface. This could indicate that the expression of this antigen requires an interaction with a nonepidermal substrate. Keratin content was consistently decreased in transformed cells when determined by radioimmunoassay, but the cytoskeletal arrangement of keratin fibers appeared normal by IIF. The functional importance of this quantitative change between normal and malignant cells is currently unexplained. Since keratin has both a cytoskeletal and differentiative function in epidermis, a decrease in keratin content could affect either of these processes. However, one cannot rule out the possibility that a decrease in antigenicity due to modifications in protein structure could contribute to the lower values obtained in the immunoassay or that other keratin molecules are expressed (for example, in the enriched region between M.W. 45,000 and 60,000) which do not cross-react with the antiserum.

The detection of a mesenchymal cytoskeletal protein by IIF was reported to occur in an epidermal cell line but not in normal epidermis (14). This could not be confirmed in our study. Although the antiserum used in our study was prepared against the CHO (hamster) intermediate filament protein while the previous report utilized antibody to mouse 3T3 intermediate filament protein, these proteins are believed to be antigenically and biochemically identical (2). A negative result in IIF cannot be considered as conclusive evidence for the absence of a specific protein. Studies on Pam 212 cells performed in collaboration with Drs. Robert Goldman and Peter Steinert have indicated that intermediate filament proteins of the mesenchymal type can be isolated in abundance from Pam 212 with appropriate extraction techniques (37). Two major proteins of approximate M.W. 55,000 and 50,000 to 53,000 are present on polyacrylamide gels and are coexpressed with the keratin proteins. The 55,000 protein from Pam 212 comigrates with the 55,000 intermediate filament protein of baby hamster kidney cell line 21, and antibody to the baby hamster kidney cell line 21 protein stains Pam 212 by IIF (37).[5] The presence of similar proteins in all Pam lines remains to be determined. The expression of both types of cytoskeletal filament systems (keratin and mesenchymal) in malignant epithelial cells could be a useful marker of aberrant expression associated with cancer. Sodium dodecyl sulfate:polyacrylamide gel patterns of urea extracts from normal and Pam cells failed to yield a characteristic qualitative change (Fig. 3), although some quantitative differences were noted. Future studies with extraction techniques designed to yield specific types of filamentous proteins may be more fruitful.

Only one *in vitro* characteristic was consistently associated with cancer in the Pam lines. Like normal epidermal cells, Pam cells have the capacity to grow under conditions of low-calcium concentration (0.02 to 0.09 mM) in the culture medium. This has also been observed for malignant cells of mesenchymal origin (1) and for transformed liver cells (33) but not their normal counterparts. When the calcium content is raised to that of standard culture medium (1.4 mM), Pam cells undergo a stratification process characteristic of epidermis *in vitro* but continue to grow rapidly, while normal cells treated in the same way cease proliferation and slough from the culture dish. Thus, Pam cells have an alteration in the pathway which inevitably leads to cell death via calcium-induced differentiation as observed for normal keratinocytes. Therefore, Pam cells can establish a balanced population consisting of continued proliferation and differentiation in the presence of 1.4 mM calcium. This characteristic has also been observed in some clones of epidermal cells treated with chemical carcinogens in medium containing 0.02 mM calcium and selected at a later time by a switch to culture medium with 1.4 mM calcium (24, 46). Since these latter cells are not tumorigenic but retain many epidermal characteristics, this alteration in differentiation may represent an early change during the process of neoplastic transformation. The nature and regulation of this difference between normal and altered cells are unknown. There could be a primary defect in the response to changes in extracellular calcium. Epidermal cells, and perhaps other stratifying squamous epithelia, appear to be unique in their responses to extracellular calcium. They grow in low-calcium environments which support the growth only of malignant cells derived from other sources (20, 21) and differentiate under conditions in which other cell types show maximum growth. Since Pam cells undergo a partial induction of differentiation in response to calcium, in that they stratify and produce squames, the initial response to changes in extracellular calcium is probably intact. Pam cells differ from normal cells in that they fail to demonstrate a proliferative block which accompanies calcium-dependent terminal differentiation in normal cells. Thus, a primary alteration in the response to a calcium related program of differentiation which is accompanied by loss of proliferative capacity in normal cells appears to distinguish Pam cells from normal keratinocytes. It seems likely this change is fundamental to the development of cancer in tissues which terminally differentiate.

In conclusion, studies with Pam cells have indicated that several criteria previously associated with cancer in other cell types, or in less-differentiated cells derived from epidermis, are not universally applicable. Criteria previously observed in some squamous cell tumors *in vivo* also cannot be used as definitive evidence of cancer *in vitro*. However, a fundamental alteration in the program of differentiation may represent a common change in all transformed epidermal cells. Future studies will be directed toward identifying specific changes (cytoskeletal, enzymatic) which may be causally associated with this biological alteration and which can be used as a marker to recognize this change.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Miriam Poirier for the keratin radioimmunoassay, Dr. Jerrold Ward for review of the tumor pathology, Dr. Bruce Casto for providing methodology for Methocel suspension, and Drs. Henry Hennings and Peter Steinert for critical review of the manuscript. The secretarial work of Margaret Green and the photographic assistance of Douglas Jones are also acknowledged.

REFERENCES

1. Boynton, A. L., and Whitfield, J. F. Different calcium requirements for proliferation of conditionally and unconditionally tumorigenic mouse cells. Proc. Natl. Acad. Sci. U. S. A., *73:* 1651–1654, 1976.
2. Cabral, F., Willingham, M. C., and Gottesman, M. M. Ultrastructural localization to 10 nm filaments of an insoluble 58K protein in cultured fibroblasts. J. Histochem. Cytochem., *28:* 653–662, 1980.
3. Carruthers, C. Differences in the urea-extracted proteins of mouse epidermis and squamous cell carcinomata determined by fluorescence microscopy. Br. J. Cancer, *28:* 36–45, 1973.
4. Colburn, N. H. The use of tumor promoter responsive epidermal cell lines to study preneoplastic progression. *In:* L. M. Franks and C. B. Wigley (eds.), Neoplastic Transformation in Differentiated Epithelial Cell Systems *in Vitro*,

[5] R. D. Goldman, P. Steinert, and S. H. Yuspa, unpublished data.

pp. 113–134. New York: Academic Press, Inc., 1979.
5. Colburn, N. H., Former, B. F., Nelson, K. A., and Yuspa, S. H. Tumor promoter induces anchorage independence irreversibly. Nature (Lond.), *282:* 589–591, 1979.
6. Colburn, N. H., Vorder Bruegge, W. F., Bates, J. R., Gray, R. H., Rossen, J. D., Kelsey, W. H., and Shimada, T. Correlation of anchorage-independent growth with tumorigenicity of chemically transformed mouse epidermal cells. Cancer Res., *38:* 624–634, 1978.
7. DeMoragas, J. M., Winkelmann, R. K., and Jordon, R. E. Immunofluorescence of epithelial skin tumors I. Patterns of intercellular substance. Cancer (Phila.), *25:* 1399–1403, 1970.
8. DeMoragas, J. M., Winkelmann, R. K., and Jordan, R. E. Immunofluorescence of epithelial skin tumors II. Basement membrane. Cancer (Phila.), *25:* 1404–1406, 1970.
9. Diaz, L. A., Weiss, H. J., and Calvanico, N. J. Phylogenetic studies with pemphigus and pemphigoid antibodies. Acta Dematovener (Stockholm), *58:* 537–540, 1978.
10. DiPaolo, J. A., and Casto, B. C. Chemical carcinogenesis. *In:* R. C. Gallo (ed.), Advances in Cancer Research: Cell Biology, Molecular and Tumor Virology, pp. 17–47. Cleveland: CRC Press, 1977.
11. Elgjo, K., Hennings, H., Michael, D., and Yuspa, S. H. Natural synchrony of newborn mouse epidermal cells *in vitro*. J. Invest. Dermatol., *66:* 292–296, 1976.
12. Elias, P. M., Yuspa, S. H., Gullino, M., Morgan, D. L., Bates, R. R., and Lutzner, M. A. *In vitro* neoplastic transformation of mouse skin cells: morphology and ultrastructure of cells and tumors. J. Invest. Dermatol., *62:* 569–581, 1974.
13. Fisher, P. B., Weinstein, I. B., Eisenberg, D., and Ginsberg, H. S. Interactions between adenovirus, a tumor promoter, and chemical carcinogens in transformation of rat embryo cell cultures. Proc. Natl. Acad. Sci. U. S. A., *75:* 2311–2314, 1978.
14. Franke, W. W., Schmid, E., Breitkreutz, D., Luder, M., Boukamp, P., Fusenig, N. E., Osborn, M., and Weber, K. Simultaneous expression of two different types of intermediate sized filaments in mouse keratinocytes proliferating *in vitro*. Differentiation, *14:* 35–50, 1979.
15. Fusenig, N. E., Amer, S. M., Boukamp, P., and Worst, P. K. M. Characteristics of chemically transformed mouse epidermal cells *in vitro* and *in vivo*. Bull. Cancer (Paris), *65:* 271–280, 1978.
16. Fusenig, N. E., Breitkreutz, D., Boukamp, P., Luder, M., Irmscher, G., and Worst, P. K. M. Chemical carcinogenesis in mouse epidermal cell cultures: altered expression of tissue specific functions accompanying cell transformation. *In:* L. M. Franks and C. B. Wigley (eds.), Neoplastic Transformation in Differentiated Epithelial Cell Systems *In Vitro*, pp. 37–98. New York: Academic Press, Inc., 1979.
17. Fusenig, N. E., and Worst, P. K. M. Mouse epidermal cell cultures. Exp. Cell Res., *93:* 443–457, 1975.
18. Green, H. Terminal differentiation of cultured human epidermal cells. Cell, *11:* 405–416, 1977.
19. Heidelberger, C. Chemical oncogenesis in culture. Adv. Cancer Res., *18:* 317–366, 1973.
20. Hennings, H., Holbrook, K., Steinert, P., and Yuspa, S. H. Growth and differentiation of mouse epidermal cells in culture: effects of extracellular calcium. *In:* I. A. Bernstein and M. Seiji (eds.), The Biochemistry of Normal and Abnormal Epidermal Differentiation, pp. 3–22. Tokyo: University of Tokyo Press, 1980.
21. Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell, *19:* 245–254, 1980.
22. Indo, K., and Miyaji, H. Qualitative changes in the biologic characteristics of cultured fetal rat keratinizing epidermal cells during the process of malignant transformation after benzo[a]pyrene treatment. J. Natl. Cancer Inst. *63:* 1017–1027, 1979.
23. Knowles, M. A., and Franks, L. M. Ultrastructure and biological markers of neoplastic change in adult mouse epithelial cells transformed *in vitro*. Br. J. Cancer, *37:* 603–611, 1978.
24. Kulesz-Martin, M. F., Koehler, B., Hennings, H., and Yuspa, S. H. Terminal differentiation assay for chemical carcinogenesis in mouse epidermal cells. Proc. Am. Assoc. Cancer Res., *21:* 120, 1980.
25. Lavin, P., and Koss, L. G. Studies of experimental bladder carcinoma in Fischer 344 female rats. II. Characterization of 3 cell lines derived from induced urinary bladder carcinomas. J. Natl. Cancer Inst., *46:* 597–614, 1971.
26. Lichti, U., Slaga, T., Ben, T., Patterson, E., Hennings, H., and Yuspa, S. H. Dissociation of tumor promoter-stimulated ornithine decarboxylase activity and DNA synthesis in mouse epidermis *in vivo* and *in vitro* by fluocinolone acetonide, a tumor-promotion inhibitor. Proc. Natl. Acad. Sci. U. S. A., *74:* 3908–3912, 1977.
27. Marchok, A. C., Rhoton, J. C., Griesemer, R. A., and Nettesheim, P. Increased *in vitro* growth capacity of tracheal epithelium exposed *in vivo* to 7,12-dimethylbenz[a]anthracene. Cancer Res., *37:* 1811–1821, 1977.
28. Montesano, R., Drevon, C., Kuroki, T., Saint Vincent, L., Handleman, S., Sanford, K. K., Defeo, D., and Weinstein, I. B. Test for malignant transformation of rat liver cells in culture: cytology, growth in soft agar, and production of plasminogen activator. J. Natl. Cancer Inst., *59:* 1651–1658, 1977.
29. Muller, H. K., and Sutherland, R. C. Epidermal antigens in cutaneous dysplasia and neoplasia. Nature (Lond.), *230:* 384–385, 1971.
30. Neugut, A. I., and Weinstein, B. The use of agarose in the determination of anchorage-independent growth. In Vitro (Rockville), *15:* 351–355, 1979.
31. O'Brien, T. G., Simsiman, R. C., and Boutwell, R. K. Induction of the polyamine-biosynthetic enzymes in mouse epidermis and their specificity for tumor promotion. Cancer Res., *35:* 2426–2433, 1975.
32. San, R. H. C., Laspia, M. F., Soiefer, A. I., Maslansky, C. J., Rice, J. M., and Williams, G. W. A survey of growth in soft agar and cell surface properties as markers for transformation in adult rat liver epithelial-like cell cultures. Cancer Res., *39:* 1026–1034, 1979.
33. San, R. H. C., Shimada, T., Maslansky, C. J., Kreiser, D. M., Laspia, M. F., Rice, J. M., and Williams, G. M. Growth characteristics and enzyme activities in a survey of transformation markers in adult rat liver epithelial-like cell cultures. Cancer Res., *39:* 4441–4448, 1979.
34. Shin, S., Freedman, V. H., Risser, R., and Pollack, R. Tumorigenicity of virus-transformed cells in nude mice is correlated specifically with anchorage independent growth *in vitro*. Proc. Natl. Acad. Sci. U. S. A., *72:* 4435–4439, 1975.
35. Slaga, T. J., Viaje, A., Bracken, W. M., Buty, S. G., Miller, D. R., Fischer, S. M., Richter, C. K., and Dumont, J. N. *In vitro* transformation of epidermal cells from newborn mice. Cancer Res., *38:* 2246–2252, 1978.
36. Stanley, J. R., Nelson, P. H., Poirier, M. C., Katz, S. I., and Yuspa, S. H. Detection of pemphigoid antigen, pemphigus antigen, and keratin filaments by indirect immunofluorescence in cultured human epidermal cells. J. Invest. Dermatol., *75:* 183–186, 1980.
37. Starger, J. M., Brown, W. E., Goldman, A. E., and Goldman, R. D. Biochemical and immunological analysis of rapidly purified 10-nm filaments from baby hamster kidney (BHK-21) cells. J. Cell Biol., *78:* 93–109, 1978.
38. Steinert, P., and Yuspa, S. H. Biochemical evidence for keratinization by mouse epidermal cells in culture. Science (Wash. D. C.), *200:* 1491–1493, 1978.
39. Steinert, P. M., Idler, W. W., Poirier, M. C., Katoh, Y., Stoner, G. D., and Yuspa, S. H. Subunit structure of the mouse epidermal keratin filament. Biochim. Biophys. Acta, *577:* 11–21, 1979.
40. Weinstein, I. B., Orenstein, J. M., Gebert, R., Kaighn, M. E., and Stadler, U. C. Growth and structural properties of epithelial cell cultures established from normal rat liver and chemically induced hepatomas. Cancer Res., *35:* 253–263, 1975.
41. Weinstein, I. B., Wigler, M., Yamasaki, H., Lee, L., Fisher, P. B., and Mufson, A. Regulation of the expression of certain biologic markers of neoplasia. *In:* R. Ruddon (ed.), Biological Markers of Neoplasia: Basic and Applied Aspects, pp. 451–471. Amsterdam: Elsevier Scientific Publishing Co., 1978.
42. Williams, G. M. Primary and longterm culture of adult rat liver epithelial cells. Methods in Cell Biol., *14:* 357–364, 1976.
43. Yuspa, S. H. Mouse epidermal cell cultures as an *in vitro* model for the study of chemical carcinogenesis. *In:* U. Saffiotti and H. Autrup (eds.), *In Vitro* Carcinogenesis: Guide to the Literature, Recent Advances and Laboratory Procedures, DHEW Publication (NIH) 78-844, pp. 47–56. Washington, D. C.: U. S. Government Printing Office, 1978.
44. Yuspa, S. H., and Harris, C. C. Altered differentiation of mouse epidermal cells treated with retinyl acetate *in vitro*. Exp. Cell Res., *86:* 95–105, 1974.
45. Yuspa, S. H., Hawley-Nelson, P., Stanley, J. R., and Hennings, H. Epidermal cell culture. Transplant. Proc., *12* (Suppl. 1): 114–122, 1980.
46. Yuspa, S. H., Lichti, U., Morgan, D., and Hennings, H. Chemical carcinogenesis studies in mouse epidermal cell cultures. *In:* I. A. Bernstein and M. Seiji (eds.), The Biochemistry of Normal and Abnormal Epidermal Differentiation, pp. 173–190. Tokyo: University of Tokyo Press, 1980.
47. Yuspa, S. H., Morgan, D. L., and Levy, J. A. *In Vitro* cultivation of a chemically induced epidermal carcinoma: establishment of three cell lines and isolation of murine leukemia virus. J. Natl. Cancer Inst., *50:* 1561–1570, 1973.
48. Yuspa, S. H., Poirier, M. C., Harness, J. R., Olson, D. R., and Steinert, P. M. Specific quantification of mouse and human keratin proteins by radioimmunoassay. Biochem. J., *187:* 281–284, 1980.

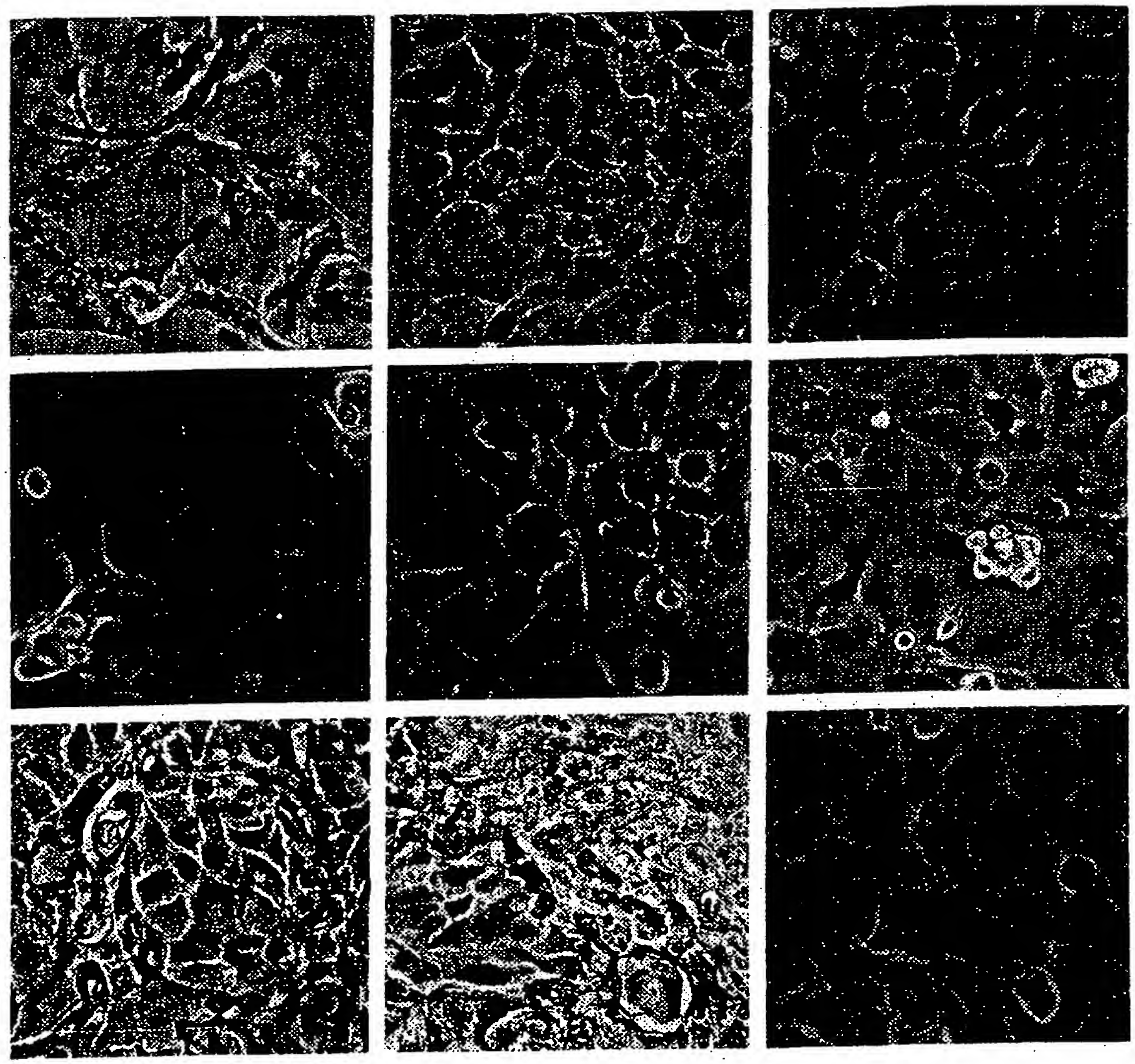
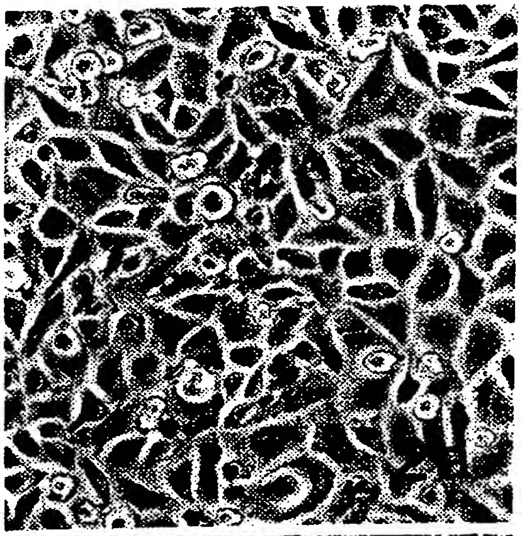
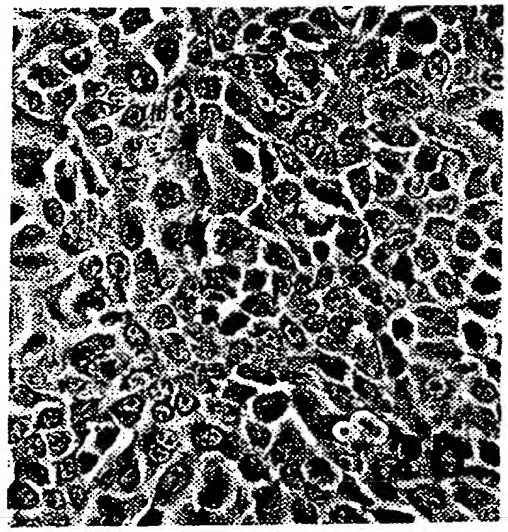
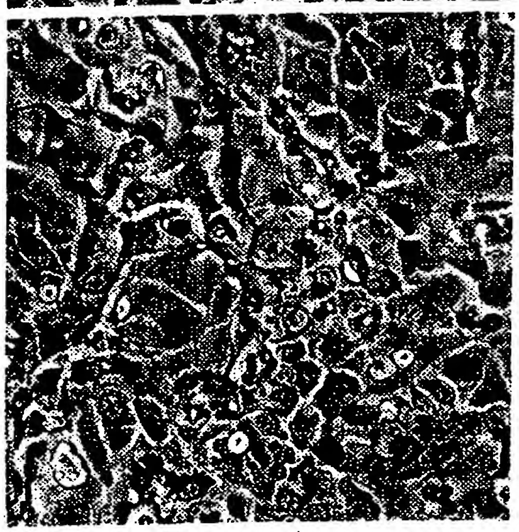
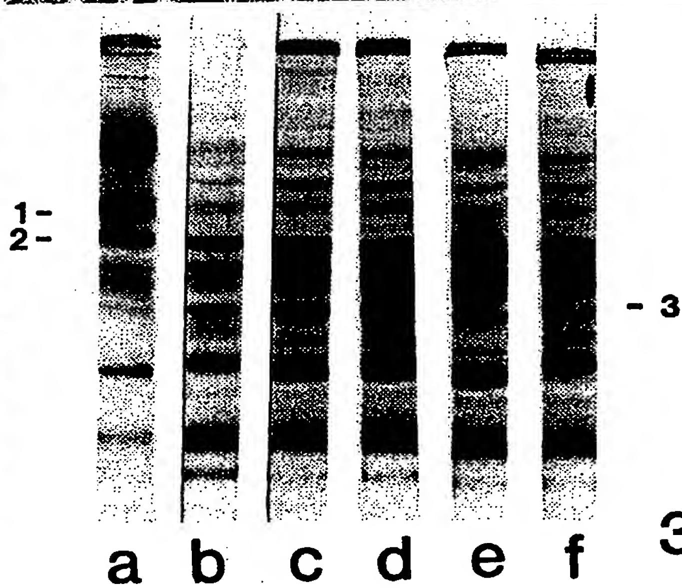


Fig. 1. Morphology of Pam lines by phase-contrast microscopy. All lines were photographed in passages 3 to 7 and have maintained morphological stability through at least 10 subcultures. Cultures were photographed at confluency, and fields which represented the most characteristic morphology of each line were selected. All lines demonstrate features of normal epidermis *in vitro* including polygonal cell shape, close cell to cell contact, granule formation, vertical stratification, and intracellular focal or diffuse keratinization. *a*, Pam 23; *b*, Pam 24; *c*, Pam 26; *d*, Pam 27; *e*, Pam 29; *f*, Pam 210; *g*, Pam 211; *h*, Pam 211, higher density; *i*, Pam 212. All photographs, × 360.









3

Fig. 2. Effect of extracellular calcium concentration on Pam 211. *a*, cells grown in medium containing 0.07 mM calcium for 1 week; *b*, 6 hr after a switch to medium containing 1.2 mM calcium; *c*, 72 hr after a switch to 1.2 mM calcium. All photographs, ×360.

Fig. 3. Sodium dodecyl sulfate:polyacrylamide gel electrophoresis of urea-soluble proteins. *a*, differentiated epidermal cells; *b*, basal cells; *c*, Pam 21; *d*, Pam 27; *e*, Pam 211; *f*, Pam 212. All were extracted with 8 M urea:0.05 M Tris-HCl buffer (pH 9.0):0.1 M 2-mercaptoethanol for 2-hr at 23° and centrifuged to remove insoluble material. The supernatant was equilibrated in cathode buffer containing 0.5% (w/v) sodium dodecyl sulfate:0.1 M 2-mercaptoethanol and heated to 95° for 2 min. A portion containing 100 to 300 µg of protein was electrophoresed for 4 hr at 2 ma per gel on 0.6- x 10-cm 9% gels. Gels were stained with Coomassie blue in 7.5% trichloroacetic acid. *1*, K_1 (keratin), M.W. 68,000; 2, K_2 (keratin), M.W. 60,000; *3*, actin, M.W. 42,000.

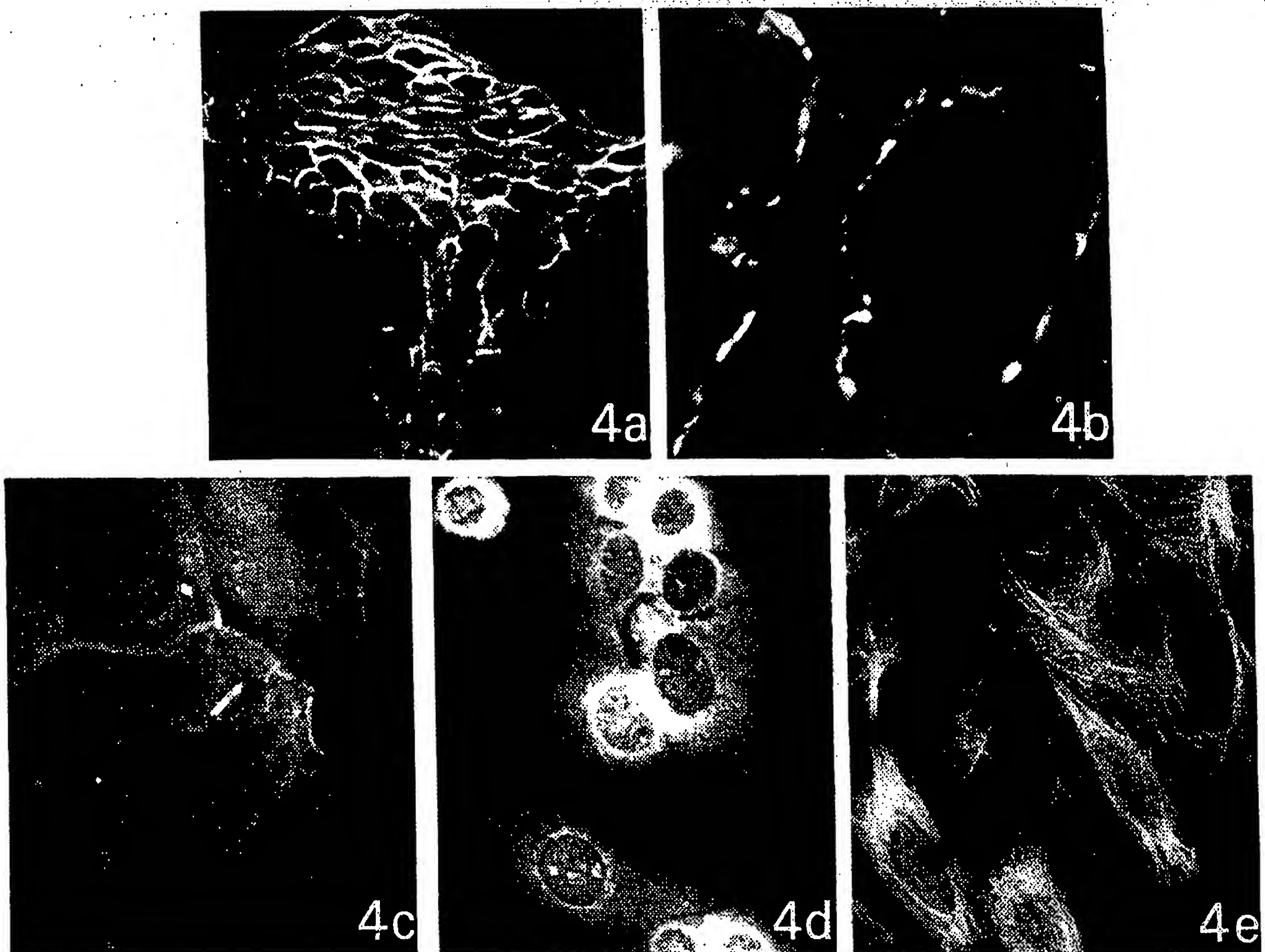


Fig. 4. IIF pattern for epidermal specific antigens in Pam tumors and cells. *a*, pemphigus antigen appears as a bright membrane fluorescence in many areas in the tumor; *b*, pemphigoid antigen is prominent as a border surrounding nests of tumor cells as the tumor epithelial cells expand into the surrounding stroma; *c*, pemphigus antigen in cultured Pam 212 cells showing membrane fluorescence seen in the larger flat cells located more superficially in the stratified colonies; *d*, pemphigoid antigen in cultured Pam 212 cells showing cytoplasmic fluorescence prominent in cells attached to the substrate; *e*, keratin proteins in Pam 212 cells *in vitro* are in a filamentous pattern radiating from the perinuclear region to the cell periphery. Filaments often appear to line up between neighboring cells. Control antisera, including 5 normal human sera and 3 normal rabbit sera, failed to reproduce any of these patterns of immunofluorescence. Magnification: a, ×260; b to e ×420.